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Review

Genomic and Postgenomic Diversity of Fungal Plant Biomass Degradation Approaches

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Plant biomass degradation by fungi is a widely studied and applied field of science, due to its relevance for the global carbon cycle and many biotechnological applications. Before the genome era, many of the in-depth studies focused on a relatively small number of species, whereas now, many species can be addressed in detail, revealing the large variety in the approach used by fungi to degrade plant biomass. This variation is found at many levels and includes genomic adaptation to the preferred biomass component, but also different approaches to degrade this component by diverse sets of activities encoded in the genome. Even larger differences have been observed using transcriptome and proteome studies, even between closely related species, suggesting a high level of adaptation in individual species. A better understanding of the drivers of this diversity could be highly valuable in developing more efficient biotechnology approaches for the enzymatic conversion of plant biomass.

Highlights

Plant biomass degradation differs strongly among fungi.

The diversity in plant biomass degradation approaches from fungi appears to be largely due to postgenomic/regulatory differences.

Despite conservation of the enzymes involved in plant biomass degradation in basidiomycete and ascomycete fungi, these two phyla have distinct regulatory systems that control plant biomass degradation.

Fungi are able to adapt the enzymes they produce to the prevailing substrate, even when this is not a substrate they naturally colonize.

The lack of sufficient biochemically characterized enzymes in many CAZy families prevents reliable genome annotation of fungi.

Composition of Plant Biomass and Its Enzymatic Degradation

Plant cell walls are the major fraction of the plant biomass and mainly consist of polysaccharides (cellulose, hemicellulose, pectin), the aromatic polymer lignin, and proteins [1]. Plant biomass also contains storage polysaccharides, such as starch, inulin, and several gums. The type, relative amount, and structure of these polymers varies considerably, depending on plant species, tissue, age, and growth season, and they consist of different monomeric building blocks. This diversity generates the need for a broad spectrum of enzymes to efficiently degrade plant biomass. The fungal enzymes involved in the degradation process have been reviewed extensively before [1–4] and will not be discussed in detail here. Plant biomass-degrading enzymes (with the exception of most feruloyl esterases) have been catalogued in the **carbohydrate active enzyme (CAZy)** (see Glossary) database (www.cazy.org) [5] in families according to their amino acid sequence similarity. Many of these families have been shown to contain multiple enzyme activities, which has led to the establishment of subfamilies for some of them, reflecting individual enzyme activities [6–8]. CAZy annotation has become a standard feature of all fungal genomes included in the MycoCosm database [9], enabling a quick comparison of the putative plant biomass degrading abilities of the corresponding species. However, the lack of available characterized enzymes in many of the CAZy families can result in mispredictions of their degradation ability (Box 1).

Efficient degradation of plant biomass requires not only a broad set of enzymes, but also efficient synergy of their activities. Synergy in the degradation of plant polysaccharides has been studied in detail, demonstrating that simultaneous activity of the enzymes leads to a higher release of monosaccharides than sequential activity [10,11]. This synergy is not restricted to the positive effect of combining a backbone and side-chain cleaving enzyme, but also occurs between different side-chain cleaving enzymes [12]. Efficient lignin degradation requires the combined action of lignin-oxidizing peroxidases and H_2O_2-generating enzymes [13]. In recent years, the benefits of enzyme synergy have also been used for biotechnological applications [14–19], demonstrating that combining enzymes from different fungal species can benefit the overall biomass hydrolysis [20].

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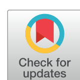

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Different Life Styles and Biotopes Require Different Approaches to Plant Biomass Degradation

Plants and plant biomass are of great importance for many fungi, as they are a major source of carbon in terrestrial biotopes. The life styles of fungi strongly affect how and to what extent they degrade plant biomass. Plant biomass-degrading fungi are found throughout the fungal kingdom, but most studies have focused on ascomycetes and basidiomycetes. Plant biomass degradation has been studied in most detail in saprobic fungi, in part because they are the species of choice for biotechnology [21]. Saprobic fungi typically have large arsenals of plant biomass-degrading enzymes (see later) to degrade their dead and sometimes already decaying substrate.

Among the wood-degrading basidiomycetes, a further distinction can be made, which was traditionally based on the visual appearance of the degraded wood [22,23]. White-rot fungi degrade both lignin and polysaccharides, using a broad enzymatic arsenal, while brown-rot fungi only modify lignin but efficiently degrade (hemi-)cellulose, using a more limited set of enzymes together with Fenton chemistry. In recent years several species have been identified that have characteristics of both white- and brown-rot fungi, which are referred to as grey-rot fungi [24].

Plant pathogenic fungi have the additional challenge of overcoming the host defense system to be able to penetrate the host tissue and gain access to their carbon source [25]. The process of plant biomass degradation after penetration can differ significantly, covering biotrophic (e.g., *Ustilago maydis* [26]) to necrotrophic (e.g., *Alternaria brassicicola* [27]) life styles. It has been shown for various plant pathogens that specific plant biomass-degrading enzymes play a role in the penetration of the plant tissue, while broader sets of plant biomass-degrading enzymes are produced during the necrotrophic/degradation phase of their life cycle [28].

Symbionts, such as mycorrhizal fungi, typically cause only limited damage to the plant cell walls, to allow the establishment of connections between the mycelium and the plant roots, but to prevent significant weakening of their host [29].

Insights from Fungal Genomes into Diversity in Plant Biomass Degradation

The different fungal life styles are often well-reflected in the set of genes encoding plant biomass-degrading enzymes present in their genomes and often show correlations to the natural substrate of the species. Examples of this are an increase in enzymes acting on cellulose and xylan in the cereal pathogen *Pyricularia* (*Magnaporthe*) *oryzae* [30], while *Botrytis cinerea* [31], a common pathogen of strawberry and tomato, contains a high number of pectinases in its genome. A comparison of growth profiles of >400 fungal species/strains on plant biomass-related substrates

Box 1. Limitation of Functional Prediction Due to Lack of a Sufficient Number of Characterized Reference Enzymes

The inclusion of CAZy annotation in all the fungal genomes hosted in the MycoCosm database [9] has led to many studies comparing the plant biomass degrading ability of fungi based on the number of genes per CAZy family. However, these predictions do not always match the experimentally determined ability. While this could in part be due to the differences in expression of the CAZy genes, another major factor is the sparsity of characterized enzymes in many CAZy families. The percentage of characterized fungal enzymes of the total number of enzymes in CAZy families related to plant biomass degradation is typically low for many families (Figure I). The characterized members often do not cover the diversity within the family, as revealed by phylogenetic analyses of several families [78,79]. This puts further questions on the functional prediction of these candidate enzymes and, with that, the overall prediction of the degrading ability of a fungus, based on genome annotation. There is an urgent need for more extensive characterization of a well-defined set of fungal reference enzymes for each CAZy family and the use of genome mining combined with amino acid sequence-based phylogeny offers a perfect starting point for the selection of these reference enzymes [79,80]. For most CAZy families, such phylogenies show distinct clades that may become subfamilies after biochemical characterization.

Glossary

ARA1: transcriptional activator in Sordariomycetes and Leotiomycetes affecting the expression of arabinanolytic genes and genes of the pentose catabolic pathway.
AraR: transcriptional activator in Eurotiomycetes affecting the expression of arabinanolytic genes and genes of the pentose catabolic pathway.
Carbohydrate active enzyme (CAZy): this refers to enzymes included in the CAZy database (www.cazy.org) that cleave and/or rearrange glycosidic bonds. Part of the families of this database contain enzymes active on plant biomass.
Carbohydrate esterase: a subsection of the CAZy database containing enzymes that hydrolyze carbohydrate esters.
CLR1/ClrA: transcriptional activator affecting the expression of cellulolytic genes.
CLR2/ClrB/ManR: transcriptional activator affecting the expression of cellulolytic and mannanolytic genes.
CreA/CRE1: main fungal regulator mediating carbon catabolite repression.
CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/endonuclease. Method of performing genome editing in many organisms, including fungi.
GaaR: transcriptional activator affecting the expression of pectinolytic genes and genes of the D-galacturonic acid catabolic pathway.
GalR: transcriptional activator affecting the expression of genes of the D-galactose oxidoreductive pathway.
Glycoside hydrolase: a subsection of the CAZy database containing enzymes that use a hydrolytic mechanism.
Polysaccharide lyase: a subsection of the CAZy database containing enzymes that use nonhydrolytic cleavage of glycosidic bonds to degrade polysaccharides.
RhaR: transcriptional activator affecting the expression of some pectinolytic genes (mainly related to L-rhamnose release) and genes of the L-rhamnose catabolic pathway.
XlnR/XLR1: transcriptional activator expression of (hemi-)cellulolytic genes and some genes of the pentose catabolic pathway.

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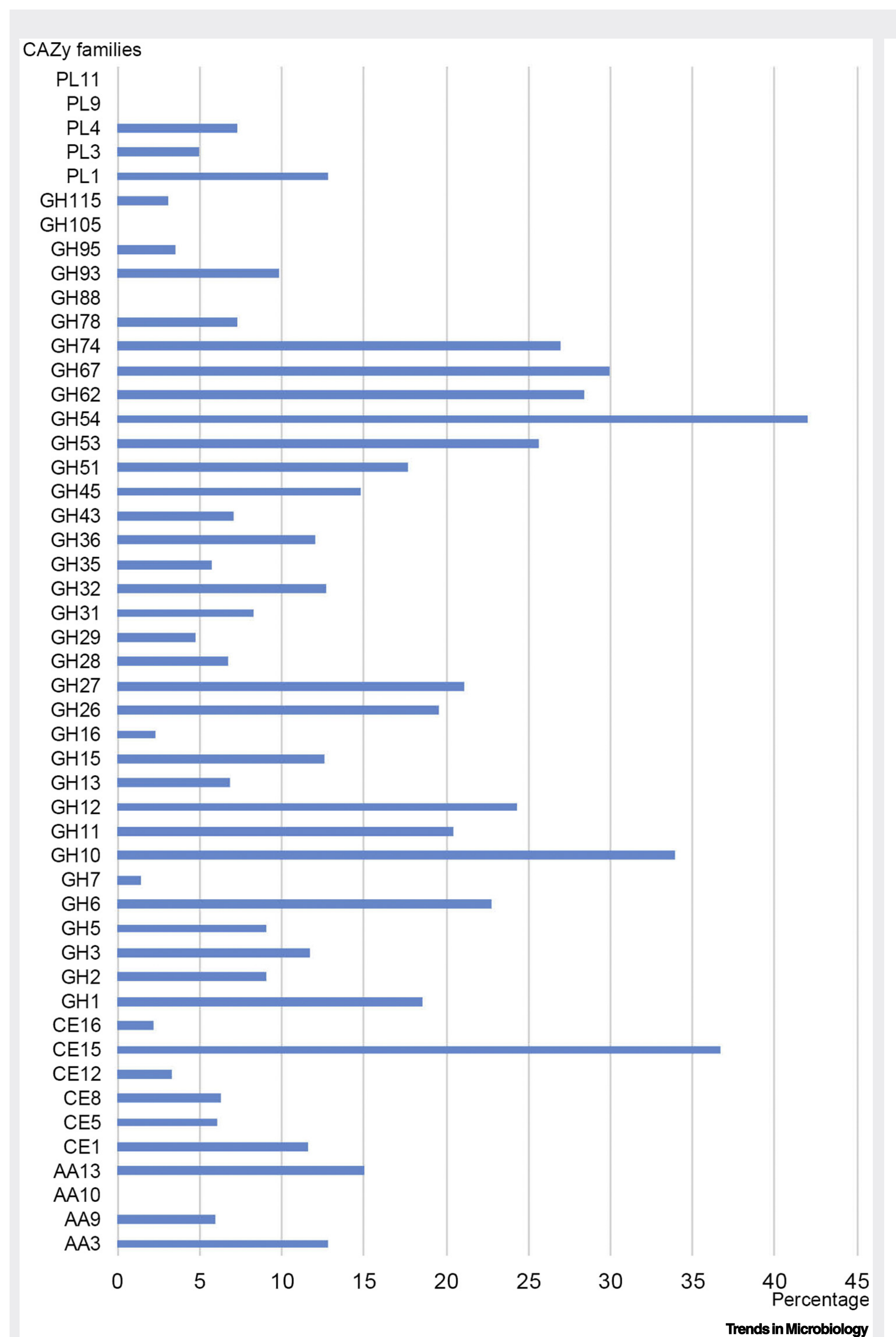

Figure I. The Percentage of Characterized Fungal CAZymes Compared with Their Total Number in the Public CAZy Database (A. Dilokpimol and X. Li, Personal Communication). Note that this database does not include most of the fungal genomes present in MycoCosm, but does contain all published characterized enzymes and is therefore a significant overestimation of the percentage of characterized enzymes. Abbreviations: AA, Auxiliary activities; CAZy, carbohydrate active enzyme; CE, carbohydrate esterase; GH, glycoside hydrolase; PL, polysaccharide lyase.

(www.fung-growth.org) with their genome content revealed that for most species, the increase or decrease in putative enzymes related to degradation of a specific polymer correlates well with the ability of a fungus to use that polymer as a carbon source. An example of that is given for two fungi in Figure 1, where the number of putative xylanolytic and pectinolytic genes correlates well with the ability of these species to grown on xylan or pectin.

There are some notable exceptions to this correlated pattern, such as the commonly used industrial cellulase producer *Trichoderma reesei* [32]. The high ability of *T. reesei* to degrade cellulose is not due to an extensive set of cellulolytic enzymes, but rather to high production of a limited set of cellulases [33].

However, while the correlation of growth and overall CAZy genome content is common, this does not apply when the number of genes in individual CAZy families are evaluated. The increasing number of sequenced fungal genomes have highlighted high diversity in the numbers of genes in specific CAZy families across the fungal kingdom. These differences suggest differences at several levels. As mentioned earlier, adaptation to specific plant polysaccharide can be observed, but also different approaches for degradation of specific polysaccharides. One of the best examples of this is degradation of pectin by different fungal species. Among the aspergilli, the *Aspergillus niger* genome contains mostly pectin hydrolases, while *Aspergillus nidulans* and *Aspergillus oryzae* have a higher number of pectin lyases and a lower number of pectin hydrolases [34]. *A. niger* acidifies its medium, while the other two species do not, which fits with the preference for acid conditions of pectin hydrolases and for neutral conditions of pectin lyases. A similar difference was observed for *Myceliophthora thermophila* and *Thielavia terrestris*, with the first being rich in pectin lyases and poor in pectin hydrolases and the opposite for the second species [35]. The functional implications of this were also confirmed, as *M. thermophila* grew better on pectin at neutral to alkaline pH, while *T. terrestris* grew best at acidic pH. However, differences can also be observed for other polysaccharides. The relative number of GH10 and GH11 endoxylanases differs strongly in fungal genomes (e.g., Table 1, *Aspergillus carbonarius* and *Aspergillus wentii*). GH10 endoxylanases are more active on branched xylans, while GH11 act mainly on nonbranched stretches in xylan [36], resulting not only in different product profiles, but also affecting the overall ability of these fungi to degrade different xylans.

These differences are also observed in closely related species that have similar habitats. Several examples of this are given in Table 1, demonstrating significant differences in numbers of genes per CAZy family between species of the same genus. *Penicillium subrubescens* stands out from the other penicillia for the strong expansions in specific CAZy families (e.g., GH11, GH28, GH43) [37].

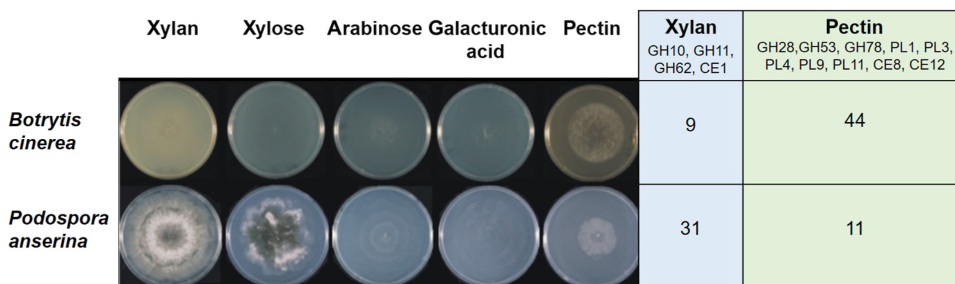

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Figure 1. Comparison of Growth of Two Ascomycete Fungi on Xylan and Pectin, Their Monomeric Components (www.fung-growth.org), and the Number of Putative Xylanolytic and Pectinolytic Genes in Their Genome [31,41,96]. The CAZy families that were taken into account in this comparison are listed.

Similarly, *Trichoderma atroviride* has a higher number of GH43 genes than the other *Trichoderma* species, while *Phanerochaete chrysosporium* has a higher number of cellulose degradation-related GH7 and GH74 genes than *Phanerochaete carnosa* (Table 1). *Talaromyces marneffei* has a reduced set of CAZy genes compared with the other species of this genus (Table 1), possibly due to its modified life style as a pathogen of mammals [38]. In fact, species-specific expansions of certain CAZy families are relatively common [4,37,39] and likely reflect an increased need for the related activity. In some cases, it has been shown that these closely related species co-inhabit the same biotope, which could suggest that the different species in the same habitat evolved to focus primarily on different components of the biomass, thereby reducing the direct competition between them. Support for this can particularly be found in fungal genera for which many species have been genome-sequenced, such as *Aspergillus* (Box 2).

Another level of diversity can be found in the fungal ability to degrade different types or components of plant biomass, differentiating the more generalist fungi, with a broad biotope and/or substrate range, from the more specialist fungi. While there is a large grey area between these two extremes, the differences can easily be seen when comparing some example species. The generalist ascomycete fungus *A. niger* possesses a broad spectrum of plant biomass-degrading enzymes [40], but the numbers per CAZy family are average compared with other fungi. In contrast, *Podospora anserina* is an ascomycete fungus that only lives in herbivore dung as a late colonizer, during which only highly complexed lignin, cellulose, and xylan are present. The genome of *P. anserina* contains a strongly increased number of cellulases and xylanases, but a much reduced number of pectinases, compared with *A. niger* and other generalist fungi [41]. This is also reflected in its ability to grow on different plant polysaccharides (Figure 1). More detailed analysis of these differences may enable predictions of genome content based on biotope range.

Postgenomic Diversity in Plant Biomass Degradation

To address the reason for the huge numbers of (putative) enzymes in fungal genomes related to plant biomass degradation, many transcriptome and proteome studies during growth of fungi on plant biomass have been reported. In most cases these studies revealed a tailored response to the composition of the biomass the fungus was exposed to, demonstrating the ability of fungi to adapt to the prevailing substrate. This adaptation was clearly shown during growth of the thermophilic ascomycete *M. thermophila* on a range of mono- and dicot substrates [42]. Only a small set of genes was induced on all plant biomass substrates, with more specific sets observed for the individual substrates. Interestingly though, the adaptation response does not seem to be restricted to substrates a fungus encounters in its natural habitat. When the wood-degrading white-rot basidiomycete *Dichomitus squalens* was grown on wood and nonwoody biomass, it also adapted efficiently to the nonwoody substrates (wheat bran and cotton seed hulls) [43], suggesting that its ability exceeds its natural substrates. This broader ability of fungi can be explored in biotechnology for the production of efficient enzyme cocktails, especially when the right substrate has been selected for the production of the enzyme cocktail. A comparison of the production of plant biomass-degrading enzymes by *P. anserina* on three plant biomass revealed that the most diverse enzyme mixture was produced on soybean hulls and this mixture resulted in higher saccharification of all three biomasses [44].

Interestingly, the tailored transcriptomic response of fungi to different substrates seems to be a major factor in the diversity of the plant biomass-degrading approach of fungi. Comparison of the response of basidiomycete fungi in 22 plant biomass-related transcriptome datasets revealed a narrow set of conserved genes that are commonly expressed during growth on plant biomass, while the majority of the genes involved in plant biomass degradation responded only in some

Table 1. Comparison of the Number of Genes in Selected CAZy Glycoside Hydrolase (GH) Families Related to Plant Biomass Degradation in Species of the Same Genus[a]

Species	*GH*	*1*	*2*	*3*	*5*	*6*	*7*	*10*	*11*	*12*	*26*	*27*	*28*	*29*	*35*	*36*	*39*	*43*	*45*	***51***	*53*	*54*	*62*	*67*	*74*	*78*	*93*	*95*	*115*
Aspergillus aculeatus		3	6	17	12	1	2	2	3	4	1	4	19	1	4	2	0	15	0	**5**	1	1	1	1	1	6	2	3	0
Aspergillus brasiliensis		3	6	21	11	**2**	2	3	**4**	5	1	4	20	1	6	2	0	11	0	4	1	1	1	1	1	8	1	2	0
Aspergillus carbonarius		3	5	16	9	1	2	0	2	3	0	3	19	1	4	1	0	13	0	3	1	1	1	1	0	5	1	2	0
Aspergillus clavatus		4	3	12	9	**2**	**4**	2	3	3	0	3	3	0	3	3	1	13	0	3	0	1	2	1	1	0	1	1	1
Aspergillus fischeri		**5**	6	19	16	**2**	**5**	**4**	**4**	5	0	5	13	0	5	3	1	20	1	2	1	1	**3**	1	2	7	**3**	2	1
Aspergillus flavus		3	8	24	15	1	3	**4**	**4**	5	1	3	21	0	**8**	3	0	21	1	4	**2**	1	2	1	0	**12**	**3**	3	**3**
Aspergillus fumigatus		**5**	6	18	14	1	**4**	**4**	3	4	0	5	12	0	5	3	1	18	1	2	1	1	2	1	**2**	5	**3**	2	1
Aspergillus luchuensis		2	6	16	9	**2**	2	2	**4**	4	1	4	19	1	5	2	0	11	0	4	**2**	1	1	1	1	7	1	2	0
Aspergillus nidulans		4	**10**	21	16	**2**	3	3	2	1	**3**	4	11	0	4	**4**	**2**	19	1	2	1	1	2	1	2	9	2	3	1
Aspergillus niger		3	6	19	10	**2**	2	2	3	4	1	5	21	1	5	2	0	11	0	4	1	1	1	1	1	8	1	2	0
Aspergillus oryzae		3	7	23	14	1	3	**4**	**4**	4	1	3	21	0	**7**	3	0	20	0	3	1	1	2	1	0	10	**3**	3	**4**
Aspergillus sydowii		3	**13**	**29**	12	1	3	2	3	2	1	**6**	11	0	4	**5**	0	**25**	1	2	1	1	2	1	1	9	**3**	3	**3**
Aspergillus terreus		3	**10**	21	**18**	**2**	**4**	4	2	**6**	0	5	8	**2**	4	**4**	**2**	21	0	4	1	1	**3**	**2**	1	4	**4**	3	**2**
Aspergillus tubingensis		3	6	18	10	**2**	2	2	**4**	4	**2**	4	19	1	5	2	0	11	0	4	**2**	1	1	1	1	7	1	2	0
Aspergillus versicolor		**5**	**13**	**28**	14	1	3	**4**	3	3	1	**6**	13	0	6	5	0	**27**	1	2	**3**	1	2	1	1	**11**	**4**	**4**	**6**
Aspergillus wentii		3	5	16	11	1	3	**4**	1	3	0	2	12	1	4	2	0	14	0	3	1	0	1	0	0	5	**4**	1	1
Meliniomyces bicolor		4	6	17	24	2	5	4	5	5	0	10	18	1	5	3	1	15	3	5	2	1	1	1	2	9	4	4	3
Meliniomyces variabilis		5	**12**	**28**	**34**	**4**	**9**	5	**8**	4	**2**	12	23	1	6	4	**5**	16	4	**9**	**3**	**3**	**2**	**2**	2	**14**	**9**	3	3
Penicillium brevicompactum		4	7	20	15	1	2	2	1	3	0	3	12	1	4	3	0	15	0	3	1	1	2	1	0	**9**	2	1	1
Penicillium canescens		6	9	22	17	1	3	**4**	1	4	0	4	15	1	**7**	2	1	21	0	3	1	1	3	1	0	**9**	**4**	**2**	1
Penicillium chrysogenum		3	7	16	14	1	2	3	1	4	1	2	5	0	4	2	2	13	0	3	1	1	1	1	0	5	2	1	0
Penicillium rubens		3	6	18	13	1	2	3	1	3	1	2	5	0	4	2	2	14	0	3	1	1	1	1	0	5	2	1	0
Penicillium digitatum		3	4	15	8	1	2	1	1	2	0	0	8	0	4	2	0	3	0	2	1	1	0	1	0	4	2	1	0
Penicillium expansum		6	10	25	13	1	2	3	1	4	1	0	9	0	4	2	0	9	0	4	1	1	0	1	0	7	2	1	0
Penicillium oxalicum		4	6	14	13	1	3	3	**5**	3	1	2	11	0	3	1	0	14	1	3	1	1	2	1	0	4	3	1	0
Penicillium subrubescens		6	11	25	**21**	**2**	**4**	3	**7**	**8**	0	**7**	**22**	**4**	**6**	**7**	**4**	**30**	1	**6**	1	**4**	**4**	**4**	0	**11**	3	**2**	**3**
Phanerochaete carnosa		2	2	11	**24**	1	5	5	1	3	0	3	4	0	4	0	0	4	1	2	1	0	0	0	2	1	0	1	1
Phanerochaete chrysosporium		2	2	10	19	1	**8**	6	1	2	0	3	5	0	3	0	0	4	2	2	1	0	0	0	**4**	1	0	1	1
Talaromyces aculeatus		**8**	**9**	**33**	**15**	1	3	2	**7**	**7**	1	5	**21**	3	**13**	7	**9**	**23**	2	**6**	1	6	**4**	2	1	**19**	**5**	**4**	1
Talaromyces funiculosus		4	**9**	**31**	**16**	1	**4**	1	**8**	**6**	1	**9**	**21**	3	5	**10**	4	**24**	2	**6**	1	**9**	**4**	**4**	1	**17**	**5**	**5**	**3**
Talaromyces marneffei		3	6	14	8	1	2	1	3	3	0	2	8	0	4	1	1	7	2	1	1	4	2	2	1	2	2	1	0
Talaromyces stipitatus		3	5	24	11	1	2	2	4	3	0	4	8	2	4	3	2	11	2	2	1	3	2	3	1	2	3	2	1
Trichoderma atroviride		**4**	10	15	11	1	2	1	4	3	0	9	6	0	1	2	2	**6**	1	**1**	0	2	2	2	1	**3**	**3**	4	1
Trichoderma harzianum		**4**	**13**	**18**	12	1	2	2	4	3	**2**	9	6	0	1	2	**3**	4	**3**	0	0	2	2	2	1	2	2	5	1
Trichoderma reesei		2	8	13	8	1	2	1	3	2	0	8	4	0	1	2	1	2	1	0	0	2	1	1	1	1	0	4	1
Trichoderma virens		2	11	**17**	12	1	2	2	4	**4**	**2**	11	6	0	1	2	1	3	2	0	0	2	**3**	2	1	**3**	1	4	1

[a]Data was based on the CAZy annotation tables of published genomes in MycoCosm [9]. Expanded gene numbers of comparisons within each genus are in bold.

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Box 2. Diversity within the Genus *Aspergillus*

Several *Aspergillus* species (e.g., *A. niger*, *A. oryzae*) have a long history of use in biotechnological applications that include degradation or modification of plant biomass, and were among the first fungal species with a sequenced genome [81]. A comparison of the first eight genome-sequenced aspergilli revealed that the genomic diversity of plant biomass degradation correlates well with their taxonomic relationship [66]. However, proteomic analysis of these species during growth on wheat bran and sugar beet pulp revealed that the enzyme sets they produce differed significantly [66]. Even enzymes for which the gene is present in all species are rarely produced by all species. An expansion of this comparison to 22 *Aspergillus* species further supported this diversity, suggesting that the different species and even strains of the same species have significant regulatory differences, resulting in the production of different enzyme sets (Figure I) [72]. Most strikingly, of the two *A. niger* strains included in this study, one abundantly produced cellulolytic enzymes, while the other produced hardly any.

More recently, genomes of all species of the *Aspergillus* genus have been sequenced, enabling an even more detailed genomic comparison. Comparative studies of species of two sections, *Nigri* [39] and *Flavi* [82], have been published revealing overall similar genome content with respect to CAZy genes related to plant biomass degradation, but in both cases also some species that have a clearly reduced number of these genes. As these differences did not have a clear correlation with growth on plant biomass substrates, this suggests that the phenotypic differences between the species are likely caused at the level of transcriptional regulation. This complete set of fungal genomes of a genus for which the taxonomic relationship of the species has been well established [83] provides a unique opportunity for detailed evolutionary studies in the development of their plant biomass-degrading approach. Experimental studies, such as those described earlier, for a larger number of species is likely to reveal the relevance of individual enzymes in relation to the efficiency of a species to degrade specific plant biomass components.

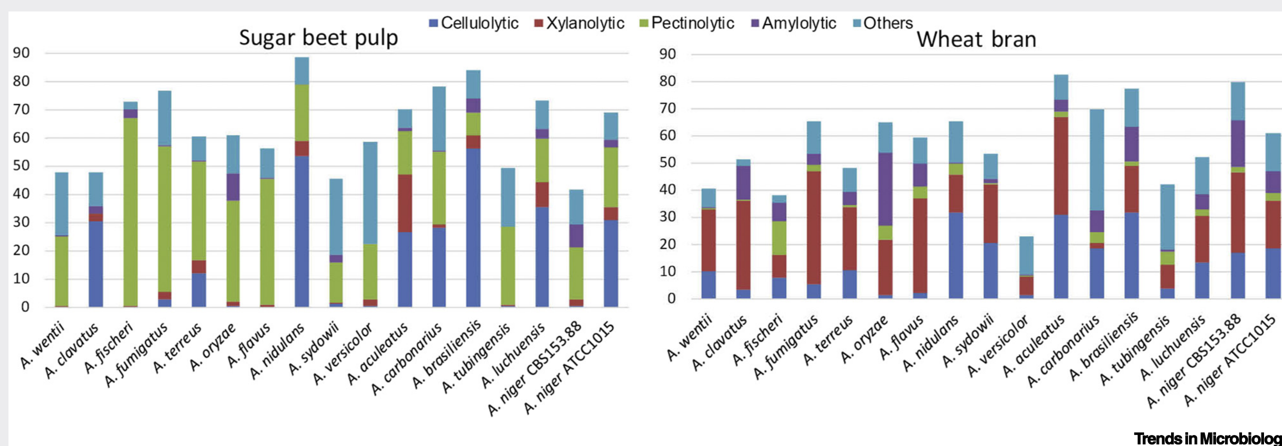

Figure I. Proteomic Response of 17 Aspergilli during Growth on Sugar Beet Pulp and Wheat Bran. The amounts of proteins associated with each enzyme activity were expressed as percentage of the amount of total extracellular proteins present in the cultures [72].

species or conditions [45]. The extensive number of studies addressing plant biomass degradation by fungi suggests that each species has a modified approach, when examined at the molecular level, both with respect to genome content of genes encoding plant biomass-degrading enzymes and the set of expressed genes in the presence of a certain (component of) plant biomass. In some cases, these differences are related to preferred environmental conditions. As mentioned earlier, comparison of *M. thermophila* with the related species *T. terrestris* demonstrated a difference in pectinolytic genes that could be directly related to their ability to degrade pectin at different pH [35]. This study also revealed significant variation in the set of genes that was expressed when these two fungi were exposed to the same substrate. A similar difference in transcriptomic and/or proteomic response has even been observed between closely related species, such as in the genus *Aspergillus* (Box 2).

Regulation of Genes Encoding Plant Biomass-Degrading Enzymes

A combination of enzymatic activities is required to degrade the complex structure of plant biomass and therefore fungi typically produce diverse enzyme mixtures during growth on plant biomass. Coregulation of genes encoding enzymes acting on the same plant polymer, but also enzymes acting on different plant polymers, has been reported across the fungal kingdom [42,46–50]. A detailed transcriptomic study in *A. niger* using both wild type and regulatory mutants grown on

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Differences observed in enzyme production of related species and strains raise the question about the stability of fungal genomes and the mechanisms that underlie this diversity. Addressing this question is important for both our basic understanding and application of fungal abilities.

Fungi are often suggested to live in a ‘state-of-war’ in natural biotopes due to competition for nutrients, but cooperation has also been reported. Does this mean that community selectivity is part of the evolutionary pressure that shapes the plant biomass degradation approach of individual fungi? Do fungi evolve to avoid direct competition and strengthen collaboration between them?

various plant biomass-related sugars, revealed extensive coregulation of genes encoding enzymes acting on different plant biomass polysaccharides [51]. Coexpression of genes acting on different components of plant biomass has also been observed for several wood-degrading basidiomycete fungi [46,48–50] and several anaerobic fungi [47]. One reason for the coregulation of these genes could be that by already producing some enzymes for a secondary polymer, the fungus is preparing to degrade it, once the primary substrate has been largely consumed. In addition, the complex structure of plant biomass may require partial degradation of some polymers in order for the fungus to gain access to the polymer it focuses on as its main substrate.

So far, the molecular regulation of plant biomass degradation by fungi has been mainly studied in ascomycete fungi, including the identification of several regulators [52]. Overall, the regulatory system typically consists of a number of transcriptional activators, each responding to their own monomeric or dimeric inducer, the general carbon catabolite repressor protein **CreA/CRE1**, and some more specific transcriptional repressors. Except for CreA/CRE1, none of these regulators are conserved across the fungal kingdom. Some (**XlnR/XLR1**, **CLR1/ClrA**, **CLR2/ClrB/ManR**) are present in most filamentous ascomycetes [52], albeit with variation in their target gene set (Box 3). However, none of the transcriptional activators of ascomycetes

The molecular mechanisms that underlie the interactions between regulators of plant biomass degradation are poorly understood. A better understanding of the regulatory network as a whole, including the role of activation of regulators and interacting proteins, will be needed to efficiently perform strain development towards optimal enzyme composition and production for different applications.

Omics technologies have strongly benefitted this field, but also exponentially widened the gap between characterized and putative enzymes. Enzymatic functions are often assigned without biochemical evidence and sometimes solely based on a CAZy family or a distant homolog. The use of these *in silico* predicted functions in describing the ability of fungus should be done carefully to avoid overinterpretation. A significant effort should be made to generate more comprehensive sets of characterized reference enzymes.

Functional annotation of fungal genomes is hampered by the high error rate in gene models. These errors are particularly common in the prediction of the start and stop codon and in the intron–exon boundaries. Reliable phylogenies of the CAZy families require them to be based on correct amino acid sequences and therefore manual curation of gene models is crucial to achieve a correct annotation.

Box 3. Functional Diversity of the (Hemi-)Cellulolytic Regulator XlnR

XlnR (also known as XLR1 or XYR1) is one of the most conserved transcriptional activators in filamentous ascomycetes [52]. It was first described as a xylanolytic regulator in *A. niger* [84] and then shown to also regulate other hemicellulolytic and cellulolytic genes [85–88]. Homologs of the gene have since then also been studied in other species, indicating differences in its set of target genes [89–93]. However, comparison of these studies were difficult due to difference in culture conditions and other experimental approaches. A comparative study of the role of XlnR in five ascomycete species using deletion strains revealed significant diversity with respect to the set of target genes and growth on D-xylose and xylan [94]. In *A. niger* and *A. nidulans*, growth of the deletion strains was only reduced on xylan but not on D-xylose, while these mutants of *Fusarium graminearum* and *T. reesei* showed reduced growth on both substrates and the deletion strain of *Pyricularia* (*Magnaporthe*) *oryzae* only grew less on D-xylose. This diversity was also reflected in the enzyme activities produced by the strains (Figure I), which was confirmed by exo-proteomics of the culture filtrates demonstrating distinct enzyme sets that decreased in the deletion mutants of the species [94]. A later study in *M. thermophila*, using the same experimental conditions, showed that the homolog in this species (Xyr1) mainly regulated xylanolytic genes and genes of pentose catabolism, but only to a small extent cellulolytic and other hemicellulolytic genes [95].

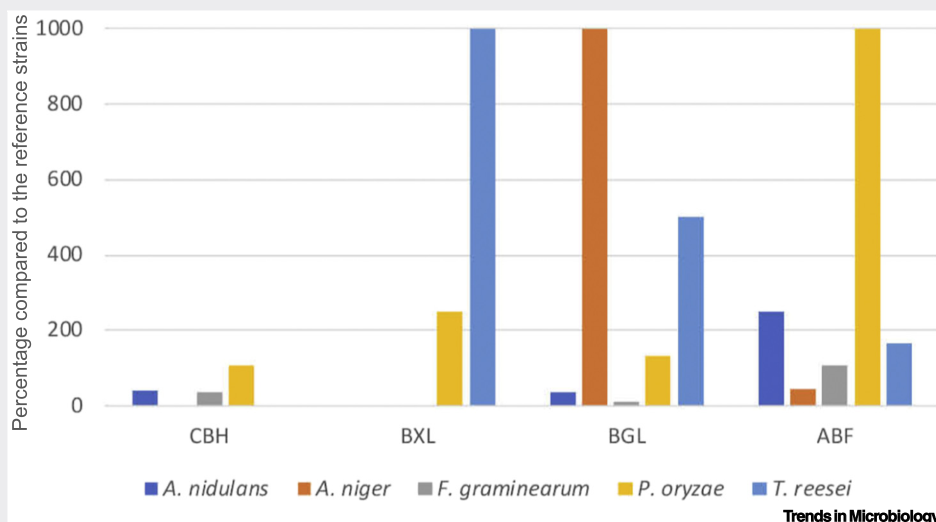

Figure I. Relative Activity of *xlnR* Deletion Strains after Growth on Xylan [94]. Abbreviations: ABF, α-Arabinofuranosidase; BGL, β-glucosidase; BXL, β-xylosidase; CBH, cellobiohydrolase.

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appear to have orthologs in basidiomycetes, despite conservation of the enzyme sets and the conditions under which the enzymes are produced (see earlier). This suggests that the regulatory systems have evolved after the split of these two phyla. Also within the ascomycetes, differences in the presence of regulators can be observed [52], including one clear example of parallel evolution. Two L-arabinose responsive transcriptional activators have been identified, one in the Eurotiomycetes (**AraR**) [53] and one in the Sordariomycetes and Leotiomycetes (**ARA1**) [54,55]. Both activators control the expression of a very similar set of genes involved in release of L-arabinose from plant biomass and the subsequent intracellular conversion of this sugar, but share no significant sequence similarity [55]. Interestingly, neither of these regulators has an ortholog in Dothideomycete fungi, suggesting that they either do not have an L-arabinose specific regulator or that the regulator with this function is not related to AraR or ARA1.

Evidence for interaction of regulators has also been reported, but the molecular basis of this interaction has remained largely unknown. As most regulators control the expression of genes encoding enzymes that release a variety of monomers (see earlier) and these monomers themselves result in the activation of other regulators, a system of interactive activation of gene expression balanced with a general repression via CreA/CRE1 can be envisioned (Figure 2), in addition to more direct interactions.

CreA/CRE1 has been shown to have a major impact on the production of plant biomass-degrading enzymes [52,56] and in fact was lost in the hypercellulolytic *T. reesei* mutant RUT-C30 [57]. While there is no evidence for direct interaction between CreA/CRE1 and other regulatory proteins involved in plant biomass degradation, it has been shown in *A. niger* that the expression level of an XlnR-regulated gene is the result of the balance between induction by XlnR and repression by CreA [58]. The induction through XlnR seems to be independent of the D-xylose concentration, while the repressing effect through CreA is clearly dependent on this [58]. In addition, it was also shown that all monomeric sugars can cause CreA-mediated repression, but that the strength of this repression depends on the individual monosaccharide, with D-glucose and D-xylose causing the strongest repression in *A. niger* and L-rhamnose the weakest [59]. This correlates with the order in which sugars are taken up by this species, when they are present as a mixture, but surprisingly this sequential uptake was not affected in a CreA mutant [60].

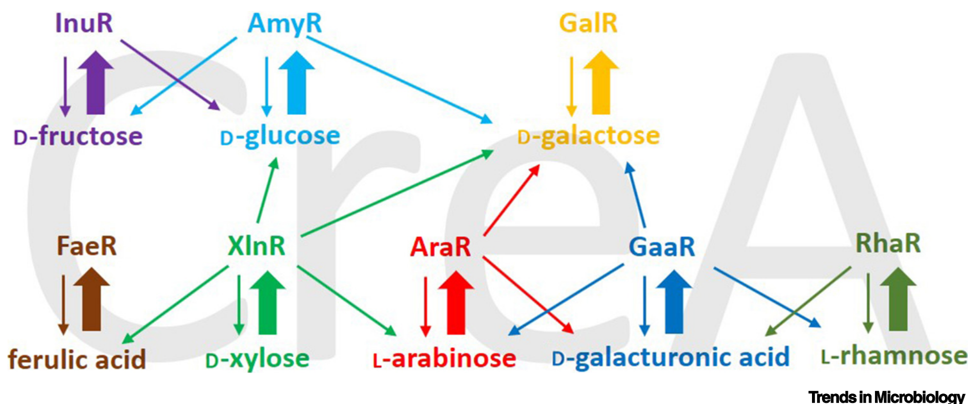

Figure 2. Model for the Indirect Interaction between Regulators Involved in Plant Biomass Degradation in *Aspergillus niger*, Based on [51]. The transcriptional activators facilitate the release of various monomers from plant biomass (indicated by thin arrows). These monomers themselves result in activation of specific regulators (indicated by thick arrows). CreA has an overruling effect in that high concentrations of any of the monomers will repress plant biomass degradation.

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Interaction between other transcription factors, not involving CreA, has also been reported. In *Neurospora crassa* and other Sordariomycetes, crosstalk between cellulose and mannan perception involved the main transcriptional regulator CLR-2, but also suggested a role for import of manno- and cellodextrins and possibly other regulators [61]. In *A. niger*, an antagonistic effect between the two pentose-related transcriptional activators, AraR and XlnR, was reported [53]. XlnR target genes are normally induced on D-xylose, while AraR target genes are induced on L-arabinose in *A. niger*. However, deletion of *xlnR* results in reduced expression of its target genes on D-xylose, while the expression of the AraR target genes increases on this sugar. Similarly, deletion of *araR* results in reduced expression of its target genes, but increased expression of XlnR target genes on L-arabinose. The molecular mechanism underlying this effect is not known, but this does not appear to be caused by direct regulation of *xlnR* or *araR* by the other regulator as there are no indications that these transcriptional activators can also act as repressors. AraR and XlnR were also shown to coregulate the D-galactose oxido-reductive pathway in *A. nidulans* together with the D-galactose responsive transcriptional activator **GalR** [62]. Similarly, degradation of pectin and conversion of the released monomers is dependent on at least three transcriptional activators in *A. niger*, **GaaR**, **RhaR**, and AraR [63]. However, these studies only scratch the surface of the interaction in the complex regulatory network that controls plant biomass degradation in these fungi. More detailed studies are required to fully reveal the molecular basis of the hierarchy and interaction of the individual regulators involved in this process. Considering the highly similar expression profiles observed for genes involved in plant biomass degradation in basidiomycetes, it can be assumed that a similar complex regulatory network also exists in these fungi. The absence of clear homologs of the ascomycete regulators in basidiomycetes (with the exception of CreA/CRE1 [64]) suggests that this network is built from different regulators, possibly from different regulator classes. The majority of the ascomycete transcriptional activators are members of the Zn_2Cys_6 class of regulators, which is the most expanded class in ascomycetes [65]. In basidiomycetes, the CCHC class of regulators is clearly expanded, which may indicate that these proteins could contain several of the plant biomass-related regulators.

Concluding Remarks

Plant biomass degradation by fungi is a highly complex process that is not only of major importance for the life of many fungi and the global carbon cycle, but also has a long history of use in biotechnological applications in the food and feed, pulp and paper, and biofuel and biochemical industries. The availability of an increasing number of fungal genomes has revealed the large diversity of genes encoding plant biomass-degrading enzymes that fungi have, as well as the large differences in these gene sets between the species. Fungal genomes also demonstrated that all species appear to have a modified approach and that model species do not really exist for this process. The classical 'model' fungus for cellulose degradation, *T. reesei*, was in fact shown to have a highly unusual approach to plant biomass degradation (see earlier) [33] and therefore does not represent the majority rule used by other fungi. It would therefore be better to avoid the word 'model species' in this field and rather use the term 'reference species', which together can reflect the diversity of this process.

The regulation of gene expression appears to have a dominant effect on diversity of plant biomass degradation approaches of fungi (see Outstanding Questions). Even related fungi with similar genome content produce highly diverse enzyme sets when grown on the same plant biomass substrate [66]. This has so far not been studied across the fungal kingdom as there are only few fungal genera with an available genome sequence for multiple species and plant biomass degradation is studied in only some of them. While an increasing number of regulators involved in this process is being identified in

ascomycete fungi, almost none have been identified in other fungal phyla, severely limiting our understanding.

Genomic and postgenomic comparisons of fungi are typically based on a single isolate of a species, as for most species only one genome sequence is available. This raises questions of whether these comparisons truly reveal differences between species, as this is largely dependent on the diversity within a species (see Outstanding Questions), and to what extent the selected strain is a good representative of the species. In most cases, no extensive comparison of strains has been performed before selection of the strain for genome sequencing. Often (e.g., *A. nidulans* [67]), the sequenced strain has been domesticated for many decades and may no longer be very similar to isolates recently obtained from natural habitats. When multiple strains were genome sequenced, clear differences were observed between them [68–71]. In addition, the production of CAZymes can also be highly strain dependent (Box 2 [66,72]), which would obviously affect the comparisons between species.

The current omics technologies, together with recent advances in genetic modification of fungi, such as **CRISPR/Cas9** genome editing [73], has opened the door to a much deeper understanding of complex biological processes, such as plant biomass degradation. The high diversity between species and strains puts severe challenges on the selection and development of strains and enzyme cocktails for biotechnological applications. The preference of industry for applications using a single fungal strain is opposite to the natural situation, where communities of fungi, bacteria, and other organisms fully degrade plant biomass (see Outstanding Questions) [74,75]. Development of novel or improved applications of fungi should therefore not ignore the synergistic value of the enzymes originating from different species [20] and also consider the more challenging, but possibly beneficial, effects of fungal cocultures [76,77].

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